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Dynamic changes in microbiome during traditional Vietnamese fish sauce fermentation revealed by metagenomics analysis

Thi Thanh Thanh Vu [a,b], Vincenzo Valentino [b], Thi Huyen Tran [a], Ngoc Nam Trinh [a], Trung Hau Nguyen [a], Khanh Hoang Nguyen [a], Francesca De Filippis [a,b,*], Sao Mai Dam [a,**]

[a] Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City, 12 Nguyen Van Bao Street Hanh Thong Ward, Ho Chi Minh City, 700000, Vietnam

[b] Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055, Portici, NA, Italy

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ABSTRACT

"Nước mắm" is a traditional Vietnamese fish sauce made from fish and salt through natural fermentation. The fish sauce fermentation process is regulated by complex metabolic activities driven by the combined effects of enzymes and microbial communities. We performed metagenomic analyses on four samples to reveal the role of specific microbes in protein degradation and flavor production within the bacterial community. Among the four analyzed fish sauce samples, *Tetragenococcus halophilus* was the dominant species in the bacterial communities. The relative abundance of *Tetragenococcus halophilus* increased after six months of fermentation, reaching its highest level at 66.07%. Protease profiling in fish sauce samples (1, 3 and 6 months of fermentation) identified various enzymes from *Tetragenococcus, Staphylococcus* and *Bacillus* species, including prolidase and Xaa-Pro aminopeptidase. These enzymes enhanced proline release, which is important for osmotic balance and flavor formation. Several abundant proteases might contribute to degrade proteins efficiently under high salinity conditions, releasing free amino acids essential for bacterial growth and flavor development. Supporting this, pangenome analysis of *Tetragenococcus halophilus* revealed a high prevalence of genes for salt stress adaptation, compatible solute synthesis, transporters and amino acid biosynthesis, supporting survival and metabolic activity in fermented fish sauce. These findings provide valuable insights into the bacterial composition and functional roles during fish sauce fermentation, and promote the exploration of potential proteolytic strains involved in flavor development to enhance production quality.

1. Introduction

Fermentation is one of the oldest techniques for preserving food, and fermented foods are well known for their distinctive flavors and improved nutritional quality (Sawant et al., 2025; Valentino et al., 2024). Various types of fermented products are obtained through desired microbial and enzymatic metabolism acting on components of different raw matrices (Marco et al., 2021). Fish sauce is a traditional Vietnamese condiment that has been produced for centuries, made by fermenting salted anchovies (*Stolephorus chinensis*) for 12 months or longer. Fish and salt are the main ingredients for producing fish sauce. The ratio of fish to salt is typically 3:1 (Ma et al., 2022). These ingredients are then fermented in wooden barrels made from *Litsea glutinosa* wood. Traditional Vietnamese fish sauce is one of the main products of the seafood industry. Approximately 40–60% of the total fish caught is utilized for fish sauce production. According to information from the Department of Processing and Market Development of Agricultural Products (Ministry of Agriculture and Rural Development), Vietnam has over 4200 fish sauce production facilities distributed along its coastline. Each year, over 498.10 million liters of fish sauce are consumed in Vietnam, with an average per capita consumption of at least 4.91 liters (General Statistics Office of Vietnam). In 2015, the Vietnamese fish sauce market was valued at about 501 million USD, with an output of >70,000 tons of fish sauce. Regarding exports, fish

* Corresponding author at: Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055, Portici, NA, Italy.
** Corresponding author.
E-mail addresses: thithanhthanh.vu@unina.it (T.T.T. Vu), vincenzo.valentino2@unina.it (V. Valentino), tranthihuyen@iuh.edu.vn (T.H. Tran), trinhngocnam@iuh.edu.vn (N.N. Trinh), nguyentrunghau@iuh.edu.vn (T.H. Nguyen), nguyenkhanhhoang@iuh.edu.vn (K.H. Nguyen), francesca.defilippis@unina.it (F. De Filippis), damsaomai@iuh.edu.vn (S.M. Dam).

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sauce is mainly consumed in Asia (approximately 54%), followed by Australia (around 18%), Europe (about 13%), and America (about 13%).

The fish microbiome is the key factor that drives the fish sauce fermentation through several metabolic processes involved in protein degradation, odor development and flavor formation. Proteolytic activity leads to the release of essential amino acids, which not only enhance the nutritional quality of the product but also function as key precursors in the biosynthesis of volatile flavor compounds (Marlida et al., 2024). Understanding bacterial communities is not only crucial for the development of fermentation processes but also for clarifying the microbial interactions that underlie high-quality products (De Filippis et al., 2017). In marine products, natural fermentation mainly depends on microorganisms originating from the raw materials and the processing environment (Han et al., 2024). Product uniqueness can be shaped by variations in raw material sources or production methods that lead to the selection of different bacterial communities during fermentation (Ohshima et al., 2019). For instance, microbiota analysis during fermentation of fish sauce obtained with the fishes *Pampus argenteus* (PA) and *Larimichthys polyactis* (LP) showed distinct microbial dynamics, with *Psychrobacter* being replaced by *Staphylococcus* after seven months in LP, while *Virgibacillus* dominated the fermentation in PA (Han et al., 2024). Indeed, also factory-to-factory variations have been highlighted. Ohshima et al. (2019) analyzed Thai fish sauce products from two factories. They identified *Halanaerobium* sp. as initially dominant taxon, but it was gradually replaced by *Lentibacillus* sp., *Halomonas* sp., and *Tetragenococcus* sp. in the first factory. In contrast, in the second factory *Peptostreptococcus* sp., *Peptoniphilus* sp., *Gallicola* sp., *Fusobacterium* sp., *Halanaerobium* sp., and *Vagococcus* sp. were found as dominant. Thus, it is necessary to study the microbiome involved in the fermentation process of "Nước mắm" to understand the metabolic contribution of each species, and may shed the light on possible strategies to improve the quality of this traditional fermented product (Wang et al., 2022). Furthermore, identification of functional traits of microorganisms linked to desired sensory properties of fermented fish sauce might drive future efforts towards isolation of potential starter microorganisms, thus promoting the standardization of the product, reducing spoilage and food waste, ensuring safety and improving the fish sauce market.

The development of next-generation sequencing (NGS) technology and its dramatic cost reduction in the last 10 years have overcome limitations linked to the study of fermented foods complex communities (Park & Kim, 2016). As a result, microbiome research is no longer exclusively culture-dependent, allowing direct analysis of genomic data from natural environments (Ranjan et al., 2016). In this work, we performed Whole Metagenome Sequencing of traditionally produced "Nước mắm" fish sauce at different timepoints, with the aim to depict the potential metabolic contribution of each species to the quality traits of this traditional and appreciated fermented product.

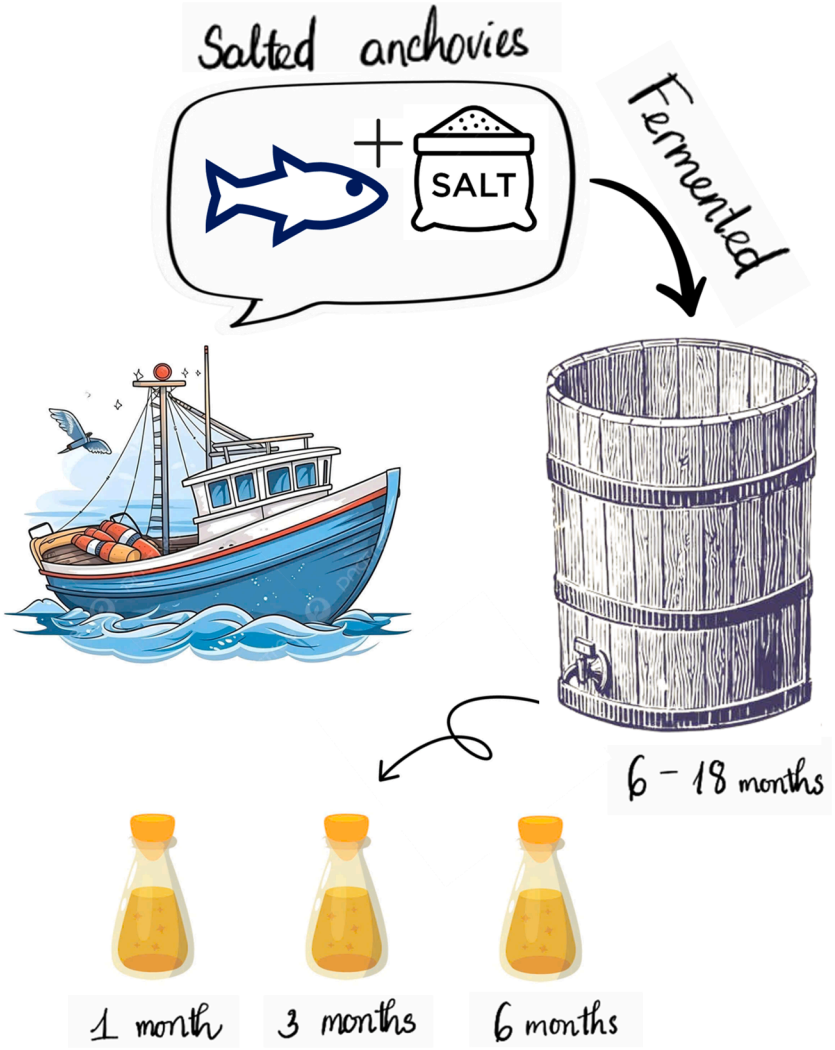

Fig. 1. Sampling procedure during fish sauce fermentation.

2. Materials and methods

2.1. Collection of samples

Salted anchovies and three fish sauce samples were collected from Phu Quoc Island, Vietnam. Salted anchovies were collected directly after landing from fishing vessels and immediately fermented. The process involves mixing anchovies with salt at a 3:1 ratio (3 parts of fish and 1 part of salt), then layering the mixture in a traditional wooden barrel (approximate capacity of 12 tons). The top layer is completely covered with salt to promote anaerobic conditions, and the mixture is then fermented at room temperature for 12 months or longer. One aliquot of about 50 mL of traditional fish sauce fermentation broth was collected after 1 month of fermentation and every three months (from day 0), up to 15 months (Fig. 1) from the drainage tap of the barrel. None of the samples showed any sign of spoilage. Samples were collected from 3 barrels and mixed before freezing. After collection, the samples were transported to the laboratory and stored at −20 °C.

2.2. Physicochemical analyses

All physicochemical parameters were analyzed in triplicate at each sampling time. Total nitrogen (TN) and ammonium nitrogen (N—NH_3) were determined using the Kjeldahl method and magnesium oxide distillation, respectively, following the procedures described by Mehmet Kilinc et al. (2006). Amino acid nitrogen (N-amino) was quantified by HPLC analysis of free amino acids with o-phthalaldehyde (OPA) according to the method of Antoine et al. (1999). Sodium chloride (NaCl) concentration was determined by argentometric titration following Lantimer (2023). Histamine content was determined at each sampling time following the procedure reported by Zhong et al. (2023).

2.3. DNA extraction and metagenomic sequencing

The salted anchovies sample was cut into small pieces for grinding, PBS 1X solution was added to the previously ground sample. The mixture was centrifuged at 5000 rpm for 2 min, then the supernatant was collected for DNA extraction. The fish sauce samples were homogenized by vortexing, using sterile medical gauze to filter and collect the liquid. Forty mL of the filtrate were centrifuged at 8000 rpm for 15 min and then the pellet was collected. The pellet was washed with 1 mL of PBS 1X buffer, transferred to a new sterile centrifuge tube and centrifuged at 15,000 rpm for 5 min. The DNeasy Powersoil pro kit (Qiagen, Germany) was used for DNA extraction according to the manufacturer's instructions. Unfortunately, the quantity of DNA extracted for the samples collected at 9, 12 and 15 months of fermentation was too low for sequencing and these samples were eliminated. The NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs, USA) was used to generate the metagenomic library. Metagenomes were sequenced using the NovaSeq 6000 (Illumina, USA) paired-end (2 × 150 PE) platform.

2.4. Metagenomic bioinformatic analysis

The quality of the raw reads was assessed through FastQC (version 0.12.1) using default options. Raw data host contamination was removed by mapping reads to the host genome (Engraulis encrasicolus and Engraulis japonicus, Supplementary Table S1) using BMTagger. Low quality reads were filtered by PRINSEQ (Schmieder & Edwards, 2011), and reads shorter than 60 bp or containing nucleotides with quality scores ≤ 5 were removed. MetaPhlAn was used to generate microbiome taxonomic profiles (Truong et al., 2017) using the default settings, and we used the 'diversity' function from the 'vegan' R package to compute the Shannon's and Simpson's (calculated as 1 – D, where $D = \sum p_i^2$, and p_i represents the relative abundance of a species). Also, high-quality reads were assembled into contigs using MEGAHIT (Li et al., 2015) with k-mer list of 21,33,55,71,81,91, and contigs shorter than 1000 bp were discarded. The coordinates and sequences of coding sequences were predicted on the contigs with MetaGenMark (Zhu et al., 2010).

To identify proteolytic enzymes, predicted genes were functionally annotated using DIAMOND BLASTP (Buchfink et al., 2021) against the MEROPS database release 12_1 (Rawlings et al., 2018) . Only matches with percent identity (pident) ≥ 80% and query coverage ≥ 80% were retained for downstream analysis. Kraken2 (version 2.0.8, options –use-name and –report) and the 'k2_plusPF' database were used to assign taxonomy to the predicted genes. The abundance of proteolytic enzymes was estimated via alignment of reads to genes encoding for proteolytic enzymes using BowTie2 (options –very-sensitive-local and –no-unal) (Langmead & Salzberg, 2012) and by calculating the RPKM (Reads Per Kilobase per Million mapped reads) value as previously reported (Mortazavi et al., 2008). Correlations between the RPKM abundance of proteolytic genes and aminoacids concentration were computed through the Spearman's ρ correlation coefficient using the function 'stat_cor' (option "method = 'spearman'") from the ggpubr R package.

The high-quality reads were mapped to the contigs using BowTie2 (options –very-sensitive-local and –no-unal). MetaBAT2 was used to process the resulting alignment files and the contigs in order to reconstruct Metagenome Assembled Genomes (MAGs) (Liu et al., 2021). MAGs were quality-assessed for completeness (≥ 50%) and contamination (< 5%) using CheckM (Parks et al., 2015). Taxonomic classification and phylogenomic tree construction were performed using GTDB-Tk (Parks et al., 2018). Genomes of *Tetragenococcus halophilus* (including one MAG recovered in this study and 74 reference genomes from the NCBI database reported in Supplementary Table S2) were functionally annotated using Prokka with default options (Seemann, 2014). Subsequently, pangenome analysis was conducted with Roary (Page et al., 2015) to compare the gene presence/absence profiles across the genomes.

All the plots were produced in a R environment (https://www.r-project.org) through the packages 'ggplot2' and 'pheatmap'.

3. Results

3.1. Physicochemical characteristics of fish sauce

The physicochemical characteristics of fish sauce are reported in Table 1 and Supplementary Table S3. The amounts of total nitrogen content ranged from 19 to 22.1 g/L. The amino acid nitrogen content increased after six months of fermentation and reached the highest value of 12.6 g/L. In contrast, the salt concentration decreased, going from 250 to 231 g/L. A total of 17 amino acids were identified, among which aspartic acid, glutamic acid, alanine, lysine, valine, and arginine were the most abundant (Supplementary Table S3). We further estimated the concentration of histamine, a biogenic amine commonly found in meat,

Table 1
Chemical composition of Fish sauce.

Samples	NaCl (g/L)	Total Nitrogen (g/L)	Ammonia Nitrogen (g/L)	Amino Acid Nitrogen g/L)	Histamine (mg/kg)
KH00	250 ± 0.38^{a}	20.8 ± 0.1^{d}	0.84 ± 0.11^{i}	4.58 ± 0.02^{i}	261.33 ± 10.02^{a}
KH01	240 ± 1.02^{b}	22.1 ± 0.09^{b}	1.1 ± 0.1^{h}	8.44 ± 0.06 g	161.67 ± 3.06^{d}
KH03	$245 \pm 4.58^{b,c}$	19 ± 0.04^{f}	$1.74 \pm 0.01^{d,e}$	10.5 ± 0.11^{e}	237.33 ± 6.66^{b}
KH06	231 ± 0.71^{d}	19.6 ± 0.15^{e}	$1.73 \pm 0.13^{d,e}$	12.6 ± 0.08^{b}	199.33 ± 4.73^{c}

Values in the same row with different letters (a–i) are significantly different ($p < 0.05$).

fish, cheese and wine products (Kimura et al., 2001). Almost all samples showed relatively high levels of histamine, ranging from 161.57 to 261.33 mg/kg, with the highest concentration found in raw fish and at the third month of fermentation. However, according to the Codex Alimentarius Standard for Fish Sauce (CXS 302–2011), the detected histamine concentrations remained within the recommended limits.

3.2. Metagenomic data processing

A total of 317.47 million raw reads were obtained from the four samples, i.e., salted anchovies (KH00) and fish sauce at one-month (KH01), three-month (KH03), and six-month (KH06) of fermentation. Reads resulting from host DNA contamination were removed by mapping against the genomes of two anchovy species, Engraulis encrasicolus and Engraulis japonicus (Table 2). Following quality filtering, 195.37 million high-quality, cleaned read were retained for assembly (Table 2) and downstream metagenomics analyses. The assembly statistics are reported in Table 3.

3.3. Taxonomic profiling of microbial communities

Fig. 2 shows the distribution of bacterial genera (Fig. 2A) and species (Fig. 2B) in salted anchovies (KH00) and fish sauce samples at the fermentation stages of one (KH01), three (KH03), and six months (KH06). At the genus level, we identified 137 genera, with 14 of them detected at relative abundances above 1% (Fig. 2A). *Psychrobacter, Tetragenococcus, Staphylococcus, Halomonas, Peptostreptococcus, Bacillus, Oceanimonas, Halobacterium* were the dominant genera in the bacterial communities of all the samples.

Three genera dominated the salted anchovies sample, i.e., *Psychrobacter* (relative abundance of 21.07%), *Peptostreptococcus* (18.07%), and *Halobacterium* (16.64%). However, their abundance decreased over the time of fermentation, reaching almost 0% after 6 months of fermentation. On the contrary, *Tetragenococcus* was underrepresented in KH00 (relative abundance = 2.24%) but increased over time, becoming the dominant genus in the bacterial communities after 3 (relative abundance 49.02%) and 6 months (relative abundance 69.49%), respectively.

At the species level, 253 bacterial species were detected, 17 of which had a relative abundance > 1%. The dominant species in fish sauce samples (one, three and six months) were *Tetragenococcus halophilus, Tetragenococcus muriaticus, Halomonas elongata, Bacillus cereus, Staphylococcus simulans, Psychrobacter celer, Oceanimonas smirnovii* (Fig. 2B). In particular, the microbial community was almost exclusively dominated by *T. halophilus* after 6 months of fermentation, that represented about 66,07% of the microbiome (Fig. 2B). As expected, the strong dominance by *T. halophilus* reduced community evenness, directly leading to a decrease in microbial diversity over time (Fig. 2 and Table 4), although we were not able to assess the statistical significance of the difference due to the limited sample size.

3.4. Prevalence of protease-encoding genes in fish sauce metagenomes

To understand the contribution of the microbial community to the specific sensory traits of fish sauce, we screened the metagenomes for their proteolytic potential. The overall abundance of proteolytic enzymes (expressed as RPKM) increased over time (Fig. 3A), and was particularly high in KH03 (RPKM = 910,870.76) and KH06 (RPKM = 935,822.69), suggesting an increased proteolytic activity over the time. More specifically, the abundance of the gene encoding C108.001 proteases increased throughout the fermentation process and suggests a key role of this protease in fish protein degradation. In our metagenomes, we found a total of 27 genes encoding C108.001 proteases, 21 of which were identified as belonging to *Staphylococcus* spp., especially *S. pseudoxylosus, S. simulans*, and *S. saprophyticus, S. nepalensis, S. epidermidis, S. arlettae* (Fig. 3B).

Furthermore, the abundance of protease family S14.001 (Clp protease acting as a serine peptidase) showed a marked increase after three (KH03, RPKM = 23,666.04) and six months (KH06, RPKM = 22,967.25) of fish sauce fermentation compared to baseline (KH00, RPKM = 7358.57). This protease breaks long protein chains releasing short peptides and free amino acids such as alanine, glycine, and leucine, depending on the substrate structure (Zeiler et al., 2013). *S. debuckii, S. simulans*, and *S. nepalensis* were the main species encoding for S14.001 proteases (Fig. 3C), while a smaller proportion originated from the *Bacillus cereus* group and the order *Lactobacillales*.

Similarly, the subfamily M24.008 encoding for exopeptidases with aminopeptidase or dipeptidyl-peptidase activity, increased significantly from raw material to 3 months and then remains constant. Genes in this subfamily were encoded mainly by *S. arlettae, S. debuckii*, and *S. nepalensis* (Fig. 3D). Therefore, our results suggest that *Staphylococcus* spp. is the main contributor to proteases over the fermentation (Fig. 3), accounting for 668 out of 951 matches (70.24% of the total proteolytic genes identified). In addition, correlations between functional gene abundance and amino acid content were assessed (Supplementary Figure 1). Only lysine and proline exhibited strong positive and significant correlations with RPKM values of proteolytic genes (R = 0.95–0.97, $p < 0.05$). Other aminoacids, i.e., glutamic acid, aspartic acid, alanine, serine, and glycine showed positive correlations with the abundance of proteolytic genes (R > 0.7), but the results were not statistically significant, possibly due to the limited sample size (Supplementary Figure 1).

Moreover, we explored the presence of proteolysis-related genes in MAGs. Overall, 37 MAGs were reconstructed from 3 samples, and no genomes were recovered from salted anchovies (Supplementary Table S4). Of these, 3 MAGs were taxonomically assigned to *Tetragenococcus*, whereas the same number was classified as Staphylococcus and Bacillus, respectively. Consistently with our previous analysis, we identified several MAGs harbouring genes encoding for proteases, mainly belonging to the genera *Staphylococcus* spp., *Tetragenococcus* spp. and *Bacillus* (Fig. 4). The MAG proteolytic profiles clustered according to the species, suggesting that different species might contribute differently to proteins degradation and, in turn, flavor development. Interestingly, protease families were shared among the species. Indeed, M24.006 and M24.008, two metalloproteinases specific for the Xaa-Pro dipeptide

Table 3
Metagenome assembly statistics.

Assembly	KH00	KH01	KH03	KH06
N° of Contigs	5855	48,869	43,776	32,203
GC (%)	41.33	46.6	46.05	44.16
N50 (bp)	1600	3591	3162	4973
Largest contigs (bp)	21,364	471,993	783,035	468,309
Total length (bp)	9578,652	135,244,119	115,131,703	104,965,812

Table 2
Summary of read preprocessing.

Samples id	Raw reads (pair)	GC (%)	Average low-quality reads	Host removal 1 (pair)	Host removal 2 (pair)	Trimmed (pair)
KH00	74,567,260	48%	0	23,911,408	23,631,340	23,168,524
KH01	92,390,934	49%	0	50,019,932	49,925,482	49,655,838
KH03	89,758,610	49%	0	62,734,914	62,577,762	62,225,666
KH06	60,749,728	44%	0	60,327,410	60,321,102	60,321,102

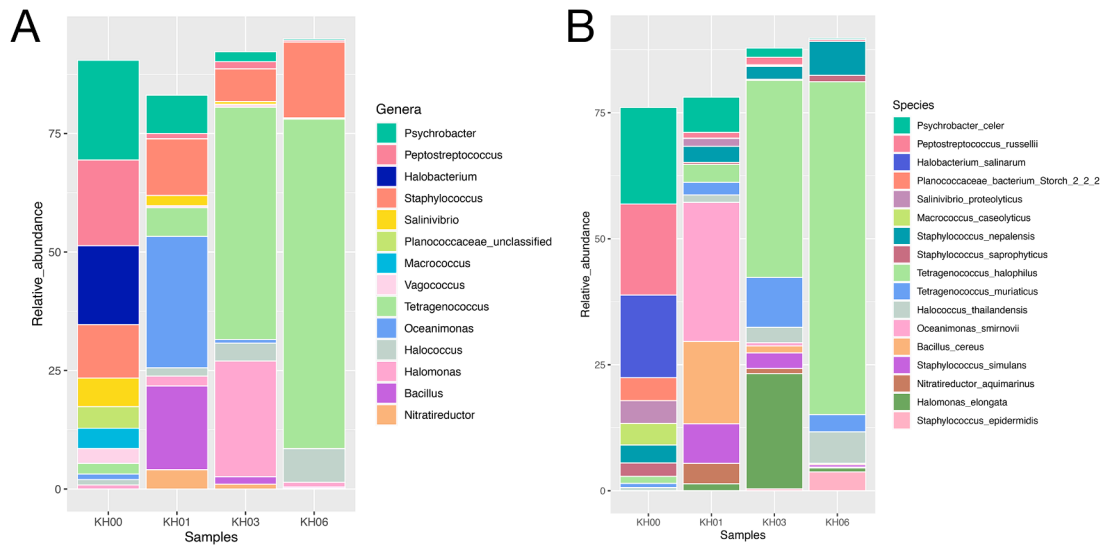

Fig. 2. Taxonomic composition of fish sauce samples at the genus (A) and species (B) levels. Only taxa with an average relative abundance >1% are shown.

Table 4
Shannon and Simpson's alpha diversity indices.

Sample	Shannon	Simpson
KH00	2.82726	0.89257
KH01	2.88051	0.87830
KH03	2.32305	0.78103
KH06	1.57583	0.55106

bond, were detected in *Staphylococcus* spp., *Tetragenococcus muriaticus* and *Tetragenococcus halophilus* (Fig. 4).

3.5. Pangenome analysis of Tetragenococcus halophilus

Pangenome provides an overview of genetic diversity for members of a phylogenetic lineage, helping to better understand genetic traits, metabolic functions, and diversity among genomes belonging to the same species (Chun et al., 2019). In this study, pangenome analysis was performed on 75 *Tetragenococcus halophilus* genomes (including 1 MAG retrieved from our samples and 74 genomes downloaded from NCBI) following gene annotation using Prokka tool. The pattern of gene presence/absence of the accessory genes was visualized in a heatmap and genomes were clustered via a hierarchical clustering (Fig. 5).

Overall, 10,050 genes were found in 75 *Tetragenococcus halophilus* genomes, including a core genome (i.e., genes present in at least 95% of the genomes (Gong et al., 2023) of 1229 genes (11.74%) and 8821 accessory genes (88.26%), highlighting the genetic diversity this species. Interestingly, the genomes clustered according to the isolation source, suggesting the genetic adaptation of strains to the different environmental niches. Interestingly, the MAG of *T. halophilus* reconstructed from sample KH06 clustered with NCBI genomes isolated from similar environments, e.g., fermented fish or shrimps (Fig. 5).

In particular, the genome cluster included our MAG and NCBI genomes isolated from fermented fish/shrimps was enriched in genes related to salt stress resistance, including *opuCC_3, yehX, yheS_2, ydeA,* and *osmC,* related to ABC transporters involved in the uptake or excretion of functional compounds (Supplementary Table S5). To regulate osmotic pressure in cells, the ABC transporter tend to accumulate compatible solutes including ions, amino acids, peptides, sugars, metabolites, and other molecules that are mainly hydrophilic (Alloing et al., 2006; Liu et al., 2015) that could withstand high osmotic pressure inside the cell to adapt to the high salt environment (Yoo et al., 2023). Moreover, some genes involved in amino acid and vitamin biosynthesis were enriched, including *proC, ubiE, PGDH, dsdA, metE,* and *bioC.*

4. Discussion

The physicochemical characteristics of fish sauce during fermentation were mainly reflected by changes in total nitrogen, free amino acid content, and salt concentration. The observed increase in ammonia nitrogen, together with a decrease in total nitrogen, reflects a dynamic transformation of nitrogenous compounds, whereby nitrogen compounds are released via hydrolysis and subsequently converted into simpler or more volatile forms. Proteolysis in fermented fish sauce is shaped by raw materials, fermentation conditions, and microbial communities at different stages, producing protein nitrogen and non-protein nitrogen compounds, including free amino acids, nucleotides, peptides, and ammonia, all of which contribute to the characteristic aroma and flavor of fish sauce (Jiang et al., 2007). After six months of fermentation, aspartic acid, glutamic acid, alanine, lysine, valine, and arginine were identified as the predominant amino acids in our samples, consistently with the amino acid composition of the Indonesian fish sauces *bekasang*, that showed lysine, phenylalanine, methionine, glutamic acid, leucine, and aspartic acid as the major amino acids upon fermentation (Ijong & Ohta, 1996).

Deciphering the microbial composition and the dynamic changes in the microbiome over the fermentation of Vietnamese fish sauce can serve as a powerful way to counteract the activity of potentially spoilage and hazardous microorganisms, thus optimizing the fermentation process and minimizing food waste. In this study, we observed that the initially dominant genera, including *Psychrobacter, Peptostreptococcus*, and *Halobacterium*, were gradually replaced by *Tetragenococcus* after six months of fermentation. *Tetragenococcus* is among the predominant microbial genera involved in the fermentation of fish sauce produced in different Asian regions (Li et al., 2022). Related studies also found that *Tetragenococcus* quickly dominated the microbial community and outcompeted other species in Chinese fish sauce over seven months of fermentation (Han et al., 2023), suggesting a key role of this taxon in fermented fish. In addition, *Tetragenococcus* spp. was isolated from Thai fish sauce (Chuea-Nongthon et al., 2017) and from brine used in cheesemaking, where it accounted for > 60% of the microbial community (Vermote et al., 2018). It is reported that *Tetragenococcus halophilus* is a halophilic species well adapted to salty environments (Holzapfel & Wood, 2014; Kobayashi et al., 2004; Link et al., 2021). Consistently with literature, *T. halophilus* had low abundance in the raw materials, salted anchovies but dominated in the sample fermented for six months. This

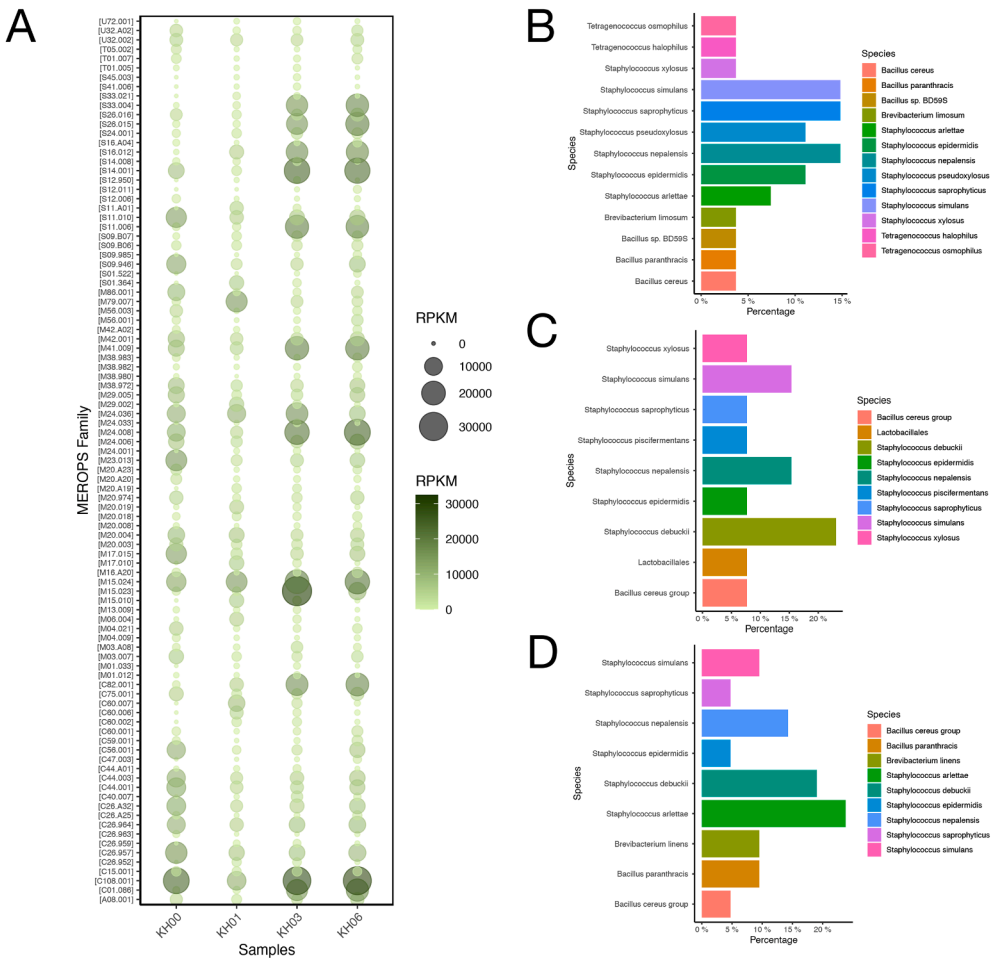

Fig. 3. Identification of genes encoding proteolytic enzymes in the fish sauce metagenome. (A) Distribution of protease gene abundance. (B) Species-level taxonomic assignment of contigs encoding C108.001 proteases. (C) Species-level taxonomic assignment of contigs encoding S14.001 proteases. (D) Species-level taxonomic assignment of contigs encoding M24.008 proteases.

dominance can be attributed to the high salinity of fish sauce, which inhibits non-halotolerant microorganisms and reduces competition for nutrients, ultimately allowing *Tetragenococcus halophilus* to become the predominant species. Based on the pangenome analysis, the adaptation of *T. halophilus* strains to high osmotic pressure environments might occur by adjusting intracellular ion concentrations and absorbing compatible substances (such as sugars and amino acids) in the fermentation medium to balance intracellular ion concentrations (Aljohny, 2015), potentially mediated by the genes *opuCC_3, yehX, yheS_2*. In addition, several genes enriched in *Tetragenococcus halophilus* genomes from salty fermented fish/shrimps were linked to the biosynthesis of essential amino acids and vitamins as stress-adaptation mechanism, including serine (contributing to sweetness; Hakimi et al., 2022), methionine (with bitter and umami taste, (Park et al., 2002) and biological cofactors such as biotin or menaquinone (vitamin K2). Therefore, the genomic comparison of newly isolated strains of *Tetragenococcus halophilus* to the species pangenome might help to identify strains resistant to osmotic pressure quickly, leading to the development of novel starter cultures for the production of fermented fish sauce with a high success rate.

Interestingly, our results are in line with previously reported data. Indeed, we observed that *Tetragenococcus halophilus* was among the potential producers of C108.001 proteases, showing the highest abundance (expressed as RPKM) in samples fermented for 3 and 6 months. This protease family encompasses enzymes that release short peptides or free amino acids, typically glycine, alanine, serine or proline, depending on the substrate (Aro et al., 2010; Denesyuk et al., 2020). The activity of this enzyme facilitates the breakdown of complex proteins, thus releasing peptides relevant to flavor formation, and enhances the metabolic potential of other microorganisms, by improving their ability to access and utilize free amino acids in natural environments, thus participating in flavour development (Teranishi et al., 1989; Xu et al., 2025; Zhu et al., 2021a).

Although *Tetragenococcus halophilus* was the dominant species during the fermentation, other minor species contributed to the proteolytic potential observed in the fermented fish sauces microbiome. Notably, several *Staphylococcus* species (including the coagulase-negative *S. simulans* and *S. debuckii*) harbour in their genomes some of the most abundant proteases found in fermented fish sauce. It has been reported that *Staphylococcus* spp. can produce protease in a high salt environment. Indeed, Casaburi et al. (2006) showed that among 18 *Staphylococcus* strains, *S. xylosus, S. saprophyticus, S. equorum, S. carnosus*, and *S. simulans*, were capable of producing proteolytic enzymes, aminopeptidases and esterases in fermented sausages from Southern Italy (Casaburi et al., 2006). Therefore, *Staphylococcus* can promote the release of amino acids through the production of proteases, which play an important role in food fermentation as flavour precursors (Zhao et al., 2024).

Proteolytic genes with prolidase activity were overrepresented in the fermented fish sauce metagenomes and, consistently, the increase in concentration of proline over the fermentation positively correlated with a higher proteolytic potential of metagenomes. Prolidase is thought

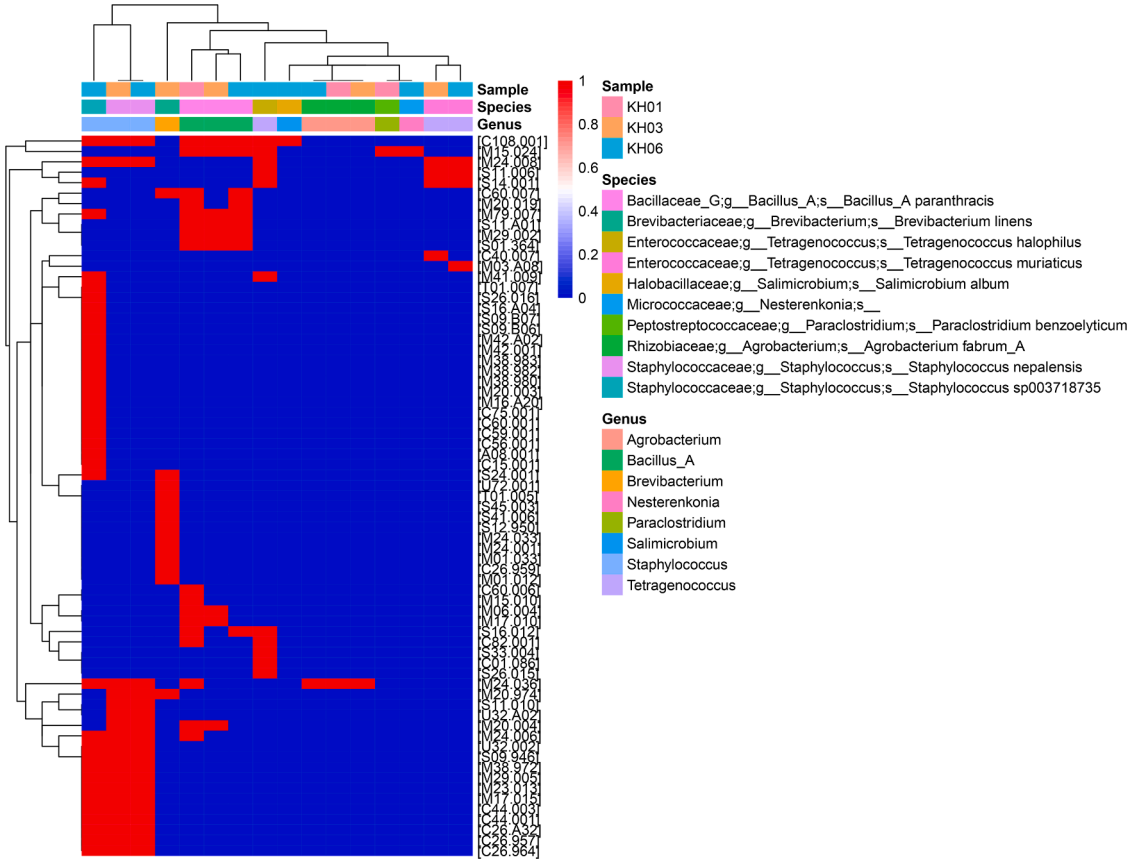

Fig. 4. Presence/absence profiles of proteolysis-related genes (from MEROPS database) in MAGs.

to function in the degradation of intracellular proteins and in proline metabolism in concert with other endopeptidases and exopeptidases (Du et al., 2005), The presence of prolidase in the metagenome, especially in *Staphylococcus* spp. and *Tetragenococcus* spp., indicates the potential of the microbiome to degrade small peptides or free proline, which are considered relevant for fish sauce flavor development (Ghosh et al., 1998; Matsushita-Morita et al., 2017; Zhu et al., 2021b). Indeed, proline is described as a main contributor to bitterness, one of the main sensorial characteristics of fish sauce, (Zhu et al., 2021a), especially when included in short peptides rather than free (Zhao et al., 2016). In addition, proline is involved in osmotic regulation (Wilk et al., 2021; Zhao et al., 2016), and osmotic pressure facilitates the release of water and soluble nitrogenous compounds from fish cells, thereby increasing the nitrogen content in the liquid phase (Hjalmarsson et al., 2007). While nitrogenous compounds are key indicators of fish sauce quality, the accumulation of amino acids contributes to product quality and flavor properties of fish sauce (Jiang et al., 2007). The combined enzymatic activity enhance the degradation of proline-containing peptide chains, leading to an increased release of free proline and contributing to the formation of characteristic flavors in fermented products. Indeed, in the food industry, prolidase and other peptidases can be added during cheesemaking to improve the flavour of the cheese by hydrolysing peptides responsible for bitter flavour (Courtin et al., 2002) and releasing amino acids that can be further metabolized, enriching the flavory bouquet of the final product (Smit et al., 2000).

Similarly, both endopeptidases (such as serine protease S14.001) and exopeptidases (including metalloprotease M24.006 and metalloprotease M15.024) were among the most abundant genes in samples fermented for 3 and 6 months. Interestingly, findings by Bu and colleagues (2022) showed that the protease activity in Thai fish sauce was higher at long fermentation times. In addition, serine protease has been also isolated from the anchovy fermented broth (Bu et al., 2022), supporting its relevance in fish sauce production. The presence of serine protease was closely related to the antioxidant activity of anchovy sauce, indicating its potential positive role not only in shaping the sensory properties of fermented fish sauce, but also in enhancing the antioxidant capacity of the product (Bu et al., 2022). Similarly, Guo and colleagues (2011) showed that metalloproteases increased over fermentation, contributing to the formation of ammonia and free amino acid (Guo et al., 2011).

Taken collectively, our results suggest that the unique sensory properties of the Vietnamese fish sauce cannot be ascribed to a specific species, but likely result from complex community dynamics where different microbial groups are selected by environmental factors such as salt addition and cooperate with their enzymes to the metabolization of fish proteins. These novel insights into the microbial community structure and dynamics might pave the way towards the identification of microbial consortia boosting the sensory properties of the fermented fish sauce. Once identified, these communities might be reproduced for the optimization of the fermented fish sauce processing technology.

5. Conclusion

This study provides comprehensive metagenomic insights into the dynamic changes in the bacterial microbiome during traditional Vietnamese fish sauce fermentation. The results indicate that *Tetragenococcus halophilus* plays a central role in the fermentation process, becoming the dominant species after six months. Functional analysis revealed that proteases derived from *Tetragenococcus, Staphylococcus*, and *Bacillus* contribute significantly to protein degradation under high-salinity conditions. Furthermore, *T. halophilus* showed extensive genetic adaptations for salt stress tolerance, compatible solute metabolism, transport systems, and amino acid biosynthesis, which explain its dominance and metabolic activity during fermentation. Overall, these results provide a foundation for the targeted selection of beneficial

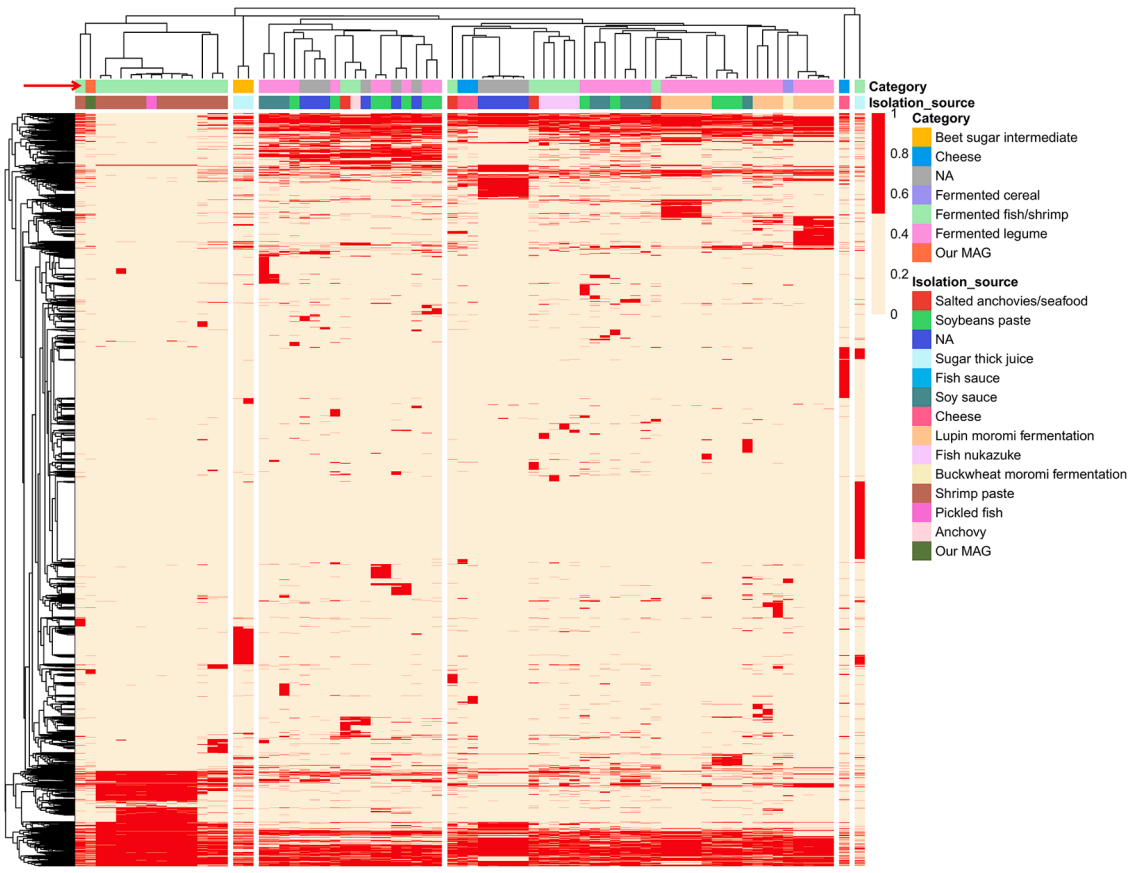

Fig. 5. Comparative gene analysis of 75 Tetragenococcus halophilus genomes, with gene presence (red) and absence (light yellow) indicated in the heatmap. Columns (i.e., genomes) are color-coded according to the isolation source.

microbes with potential applications as starter cultures to optimize fermentation efficiency, control flavor formation, and enhance the production quality and consistency of traditional fish sauce. Although our study paves the way towards the fine-tuning of microbial consortia for the optimization of fish sauce production and flavory properties, some issues should be addressed in future studies. Indeed, larger sample sizes including technical replicates, as well as integration with metabolomics and physico-chemical data (e.g., enzyme activities, volatile organic compounds), are needed to confirm our results and to better dive into the microbial dynamics occurring during fish sauce fermentation and their influence on product characteristics. Furthermore, the safety (e.g., lack of virulence and antimicrobial resistance traits) of the species described here as most relevant for flavor development must be rigorously assessed.

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Ethical statement

Not applicable.

CRediT authorship contribution statement

Thi Thanh Thanh Vu: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis. **Vincenzo Valentino:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Formal analysis. **Thi Huyen Tran:** Writing – review & editing, Formal analysis. **Ngoc Nam Trinh:** Writing – review & editing, Formal analysis. **Trung Hau Nguyen:** Writing – review & editing, Formal analysis. **Khanh Hoang Nguyen:** Writing – review & editing, Formal analysis. **Francesca De Filippis:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition. **Sao Mai Dam:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2026.102125.

Data availability

The raw reads of the four samples are available in the NCBI Sequence Read Archive database under accession number PRJNA1332412.

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